

## Specific Amplification of *Rickettsia tsutsugamushi* DNA from Clinical Specimens by Polymerase Chain Reaction

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**Polymerase chain reaction (PCR) was used to detect *Rickettsia tsutsugamushi*-specific DNA in clinical specimens. The primer pair used for PCR was designed from the nucleotide sequence of the gene encoding the 56-kDa antigen of the Gilliam strain. These primers led to a 78-bp fragment by amplifying the genomic DNAs from five serovariants, i.e., the Gilliam, Karp, Kato, Kawasaki, and Kuroki strains of *R. tsutsugamushi*, and also the DNA from blood clots of patients with scrub typhus, even at the early stage of onset of the disease. This indicates that this method is suitable for the diagnosis of scrub typhus.**

About 700 to 900 patients per year in Japan suffer from tsutsugamushi disease, a rickettsiosis transmitted by the chigger mite. Microimmunofluorescence and immunoperoxidase techniques are generally used to diagnose this disease. However, diagnosis is sometimes difficult in the early stage of the illness, when the antibody titers are not yet high enough to be detected.

Recently, the application of polymerase chain reaction (PCR) to the diagnosis of some infectious diseases has been reported. This method is excellent in circumstances in which immunological techniques or isolation of the causative agent is difficult. In tsutsugamushi disease, PCR showed rickettsial infection during the acute rickettsemia phase, which occurs before the antibody titer increases. Thus, diagnosis will be possible in the early stage of the illness. Here, we describe the use of PCR in diagnosing tsutsugamushi disease.

*Rickettsia tsutsugamushi* Gilliam, Karp, Kato, Shimokoshi, Kawasaki, and Kuroki strains used in our previous studies (4, 6, 7), *R. rickettsii* Bitterroot strain from Denka Seiken Co., Tokyo, Japan, and *R. sibirica* ATCC VR-151 donated by N. Tachibana, Miyazaki Medical College, were propagated in L929 cells as described previously (4, 7). Monolayers of infected cells in a 200-ml culture bottle were homogenized with a Dounce homogenizer (Kontes Glaso Co., N.J.) in 5 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA (TE buffer), and the DNA was extracted from homogenate supernatants obtained after centrifugation at  $300 \times g$  for 10 min. In some experiments, Gilliam strain rickettsiae purified by Percoll density gradient centrifugation (5) were suspended in a small amount of TE buffer and used for DNA preparation. Blood clots obtained from patients with tsutsugamushi disease were stored at  $-80^{\circ}\text{C}$  until use. From portions of the clots, rickettsiae were isolated in mice or L929 cells, and the rickettsial serotype was identified with strain-specific monoclonal antibodies as described previously (1). DNA was extracted from blood clots which corresponded to about 0.5 ml of blood and which had been homogenized in 1.0 ml of distilled water with a mortar.

To extract DNA preparations from these samples, the rickettsial suspensions or blood clot homogenates were mixed with 1/10 volume of 10% sodium dodecyl sulfate (SDS) (final concentration of SDS, 1%) and incubated at  $4^{\circ}\text{C}$  for 16 h. After the addition of 1/10 volume of 10-fold-concentrated TE buffer, the mixture was further incubated with  $3 \times$  crystallized chicken egg white lysozyme (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 2 mg/ml for 30 min in an ice bath and then with proteinase K (Merck & Co., Inc., Rahway, N.J.) at a final concentration of 0.2 mg/ml for 1 h at  $55^{\circ}\text{C}$ . The DNA in this lysate was purified by three extractions with an equal volume of a phenol-chloroform (1:1) mixture, followed by precipitation with 2 volumes of ethanol and resuspension in 50  $\mu\text{l}$  of TE buffer. A pair of primers (primer 1, ATAGAATTGGGTGAG GAAGGAGGATTAGAG; primer 2, ACCAGTAATCATTC CTCCAACGATTCCAAC) for PCR were synthesized by an Applied Biosystems DNA synthesizer. Primer 1 was the nucleotide sequence corresponding to 10 amino acids at the N terminus of a mature 56-kDa protein in *R. tsutsugamushi* (2), and primer 2 was complementary to the region 18 bp downstream from the first primer. These primers amplified a 78-bp fragment. The PCR amplification mixture (total volume, 50  $\mu\text{l}$ ) contained 1.5 mM  $\text{MgCl}_2$ , 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.001% (wt/vol) gelatin, 200  $\mu\text{M}$  (each) dATP, dCTP, dGTP, and dTTP, 1  $\mu\text{M}$  each primer, 1.25 U of AmpliTaq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), and 5  $\mu\text{l}$  of template DNA. The mixture was placed in a thermal cycler (Perkin-Elmer Cetus), and the temperature was denatured at  $94^{\circ}\text{C}$  for 30 s, annealed at  $57^{\circ}\text{C}$  for 2 min, and then chain extended at  $70^{\circ}\text{C}$  for 2 min. This cycle was repeated 30 times. In some experiments, 5  $\mu\text{l}$  of the amplified mixture was added into a fresh PCR reaction mixture and further amplified through 30 cycles (a total of 60 cycles). The amplified sample (5  $\mu\text{l}$ ) was electrophoresed in an 8% polyacrylamide gel, and the DNA bands were stained with 0.5  $\mu\text{g}$  of ethidium bromide per ml. For Southern blotting analysis, the 78-bp DNA probe labeled with digoxigenin was prepared by incorporating digoxigenin-11-dUTP during amplification of rickettsial DNA (Gilliam strain) with the primers. DNA obtained by amplification of clinical specimens was electrophoresed, transferred to a nylon mem-

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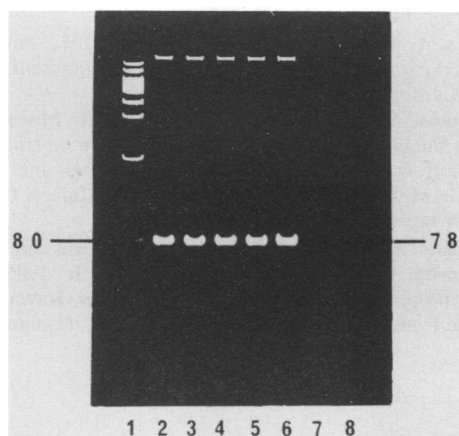


FIG. 1. Polyacrylamide gel electrophoresis of amplified DNA by PCR after 30-cycle amplification with template DNA from the strains Gilliam (lane 2), Karp (lane 3), Kato (lane 4), Kawasaki (lane 5), Kuroki (lane 6), and Shimokoshi (lane 7) and L929 cells (lane 8). Lane 1, restriction endonuclease digests of plasmid pHY as size markers (Takara Shuzo Co., Ltd., Kyoto, Japan). The sizes of the fragments are 4,870, 2,016, 1,360, 1,107, 926, 658, 489, 267, and 80 bp. Numbers on the figure are sizes (in base pairs).

brane by electrophoresis, and hybridized with the digoxigenin-labeled DNA probe, under highly stringent conditions in solution containing 0.02% SDS and  $5\times$  SSC ( $1\times$  SSC is 0.15 M NaCl and 0.015 M sodium citrate) at  $68^{\circ}\text{C}$  for 10 h. After hybridization, the membrane was washed twice with  $0.1\times$  SSC containing 0.1% SDS at  $68^{\circ}\text{C}$  for 30 min and allowed to react with alkaline phosphatase-conjugated antidigoxigenin antibody. Detailed DNA labeling with digoxigenin and the detection of DNA bands containing the drug were performed according to the manufacturer's instructions for the Genius DNA Labeling and Detection kit (Boehringer GmbH, Mannheim, Germany).

After the PCR reaction with the primers, the predicted 78-bp DNA fragment was identified as a sharp band in polyacrylamide gel electrophoresis, by using template DNA from the Gilliam, Karp, Kato, Kawasaki, and Kuroki strains, but no amplified band was seen when the DNA from the Shimokoshi strain was used (Fig. 1). The enzymatic amplifications were not seen in the tests with the DNA of *R. rickettsii*, *R. sibirica*, *Escherichia coli*, *Proteus vulgaris*, and *Proteus mirabilis* (data not shown) and with that of the host cell line (L929). These results indicate that amplification with the primers is specific for *R. tsutsugamushi* among the five antigenic variants. In our preliminary tests of PCR, we ascertained that amplified DNA was detectable when more than 1 ng of genomic DNA from the purified rickettsia was used as a template.

We also tested PCR in the diagnosis of tsutsugamushi

TABLE 1. Patient blood used for PCR

Patient no.	Age	Days after onset	Serotype of isolated rickettsia <sup>a</sup>
1	69	1	Kawasaki
2	39	7	Karp
3	52	9	Karp

<sup>a</sup> Serotype of rickettsia isolated was identified by an immunofluorescence technique with strain-specific antibodies as described previously (6).

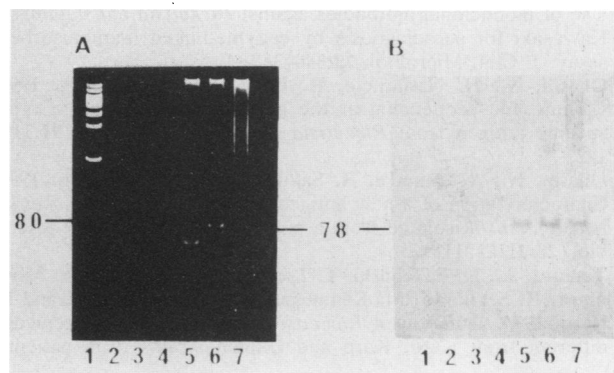


FIG. 2. (A) Electrophoresis of amplified DNA by PCR after 60-cycle amplification with the template DNA from blood from healthy humans (lanes 2 to 4) and patient blood (as shown in Table 1) (lanes 5 to 7). Lane 1, molecular size markers as described in the legend to Fig. 1. (B) Southern blot of DNA from panel A with amplified digoxigenin-labeled Gilliam 78-bp DNA probe. Numbers on the figure are sizes (in base pairs).

disease. The blood of patients from which Kawasaki- or Karp-type rickettsiae were isolated was used (Table 1). In PCR tests of the DNAs from the blood of these three patients, weakly stained bands were seen after a 30-cycle amplification (data not shown). However, by an additional 30-cycle amplification (a total of 60 cycles), clear bands were observed at the position of 78 bp, together with other minor bands (Fig. 2A). There were no detectable bands in the amplification tests of DNA from the blood of three healthy donors. In the hybridization test with the amplified digoxigenin-labeled Gilliam 78-bp DNA probe, only the 78-bp bands from the patient specimens hybridized, indicating that the 78-bp bands are DNA fragments specific to rickettsia (Fig. 2B).

As described above, we amplified the small amount of rickettsial DNA in patient blood by PCR. The results described above may be because the amplified sequence, 78 bp, is short, which is a benefit for rapid and correct polymerization even through a 60-cycle amplification. The primers used here were designed from the DNA sequence corresponding to the N-terminal area of the 56-kDa protein in the Gilliam strain, which we previously described (2). DNA of *R. tsutsugamushi* of the Gilliam, Karp, Kato, Kawasaki, and Kuroki strains was amplified by PCR with these primers. This indicates that the nucleotide sequence of the primer regions is well conserved among these strains. The DNA from the Shimokoshi strain was not amplified by PCR, indicating that these primers are not appropriate to the DNA of this strain. However, infection with Shimokoshi-type rickettsiae (5) is very rare, and almost all rickettsiae isolated from the patients with tsutsugamushi disease in Japan belong to the other five serotypes (3, 6, 7). Therefore, PCR with the primers described above is practical for diagnosis in Japan. Furthermore, the applicability of this method to clinical specimens from acute-phase patients indicated its usefulness in the diagnosis of tsutsugamushi disease.

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